00-25

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Darrell C. Conklin, Zeren Gao
Serial No.	:	09/819,136
Filed	:	March 27, 2001
For	:	MULTI-DOMAIN PROTEINASE INHIBITOR
Examiner	:	Ramirez, Delia M.
Art Unit	:	1652
Docket No.	:	00-25
Date	:	January 5, 2005

Declaration Under 37 C.F.R. § 1.131

Sir:

We, Darrell C. Conklin and Zeren Gao, hereby declare as follows:

1. We are the inventors of the above-referenced patent application (the "Patent Application").

2. All of the work described herein was performed in the United States.

3. Prior to March 24, 2000 we conceived of a polypeptide comprising SEQ ID NO:2 of the Patent Application and fragments thereof, a polynucleotide encoding a polypeptide of SEQ ID NO:2, and a protein produced by culturing a host cell transformed with an expression vector comprising a polynucleotide encoding a polypeptide of SEQ ID NO:2. The polypeptide of SEQ ID NO:2 is identified in the Patent Application as "zkun6."

4. Attached hereto as Exhibit 1 is a copy of a nucleotide sequence prepared by one of us (Zeren Gao) prior to March 24, 2000. Dates on Exhibit 1 have been intentionally obscured. The sequence shown on Exhibit 1 includes the sequence shown as SEQ ID NO:1 in the Patent Application. Nucleotide number 1 of SEQ ID NO:1 corresponds to nucleotide number 16 shown in Exhibit 1.

5. Attached hereto as Exhibit 2 is a copy of an amino acid sequence prepared by one of us (Darrell C. Conklin) prior to March 24, 2000. Dates on Exhibit 2 have been intentionally obscured. This sequence is the same as the amino acid sequence shown in SEQ ID NO:1 and SEQ ID NO:2 of the Patent Application.

6. Attached hereto as Exhibit 3 is a copy of a report prepared by one of us (Darrell C. Conklin) prior to March 24, 2000. Dates on Exhibit 3 have been obscured. This report is entitled, "Multi-domain proteinase inhibitor zkun6." Zkun6 is described in

Exhibit 3 as a 548 amino acid, multi-domain, secreted protein having seven domains, designated A through G. These domains include a four-disulfide core proteinase inhibitor domain (domain B, amino acids 33-75), a follistatin-type proteinase inhibitor domain (domain C, amino acids 93-157), a kunitz proteinase inhibitor domain #1 (domain E, amino acids 299-351), and a frzb domain C-terminal domain (domain G, amino acids 412-548). As stated in the report, these domain boundaries should be considered approximate, and may vary by +/- 5 residues.

7. From a time prior to March 24, 2000, we worked diligently with Gary E. Parker, a patent agent at ZymoGenetics, Inc., to constructively reduce the invention to practice by filing a provisional patent application, Serial No. 60/193,642, on March 31, 2000.

8. We further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true.

Darrell C. Conklin

January 6, 2005
Date

Zeren Gao

Jan. 07, 2005
Date

EXHIBIT 1

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-rw-r--r--    1 gaoz     staff        2275                huzkun6final.seq
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;combined sequence from zkun6-e2906640 and z25770g3e6+e7 andzkun6-i1678559.seq
;coding region: 391-2037
;mature start: 448
HUZKUN6
GAATTCGGCACGAGGGTGACCCTCATGGCCAGTGGCTCTGTGCTCATGGGCCTCTGGCCCCTCCCCAACCTCCTCCCCTC
TGCCCTGTGCTGACCAGGGCCTGGGAGCCCCCGCACGGTTCAGACAGAGGGGCCAGGCTGAAGCTGGAGAGGAACCAGCG
TCACACAGACGGCCTCTGAGAACTTGGAGACCCCGTTACCCACCCAGCAGGGGTGTCAGGACAAGCATCTGCTGCAGGCT
TCAGCCTCAGGGGCAAAAGGGAGCCCCGGGGTCCTGGTGGGGGCACCGACCACAGGCCCGGAGGGTGGATGCCTGCAGGA
AGCTGGGCTCTGTGGAGCCCGAGGAGGGGCTGGTGGCCACACCCCCCGGCCCCCTGGCTCGGCGGCCCTCATGCCCGCCC
TACGTCCACTCCTGCCGCTCTTGCTCCTCCTCCGGCTGACCTCGGGGGCTGGCTTGCTGCCAGGGCTGGGGAGCCACCCG
GGCGTGTGCCCCAACCAGCTCAGCCCCAACCTGTGGGTGGACGCCCAGAGCACCTGTGAGCGCGAGTGTAGCAGGGACCA
GGACTGTGCGGCTGCTGAGAAGTGCTGCATCAACGTGTGTGGACTGCACAGCTGCGTGGCAGCACGCTTCCCCGGCAGCC
CAGCTGCGCCGACGACAGCGGCCTCCTGCGAGGGCTTTGTGTGCCCACAGCAGGGCTCGGACTGCGACATCTGGGACGGG
CAGCCCGTGTGCCGCTGCCGCGACCGCTGTGAGAAGGAGCCCAGCTTCACCTGCGCCTCGGACGGCCTCACCTACTACAA
CCGCTGCTATATGGACGCCGAGGCCTGCCTGCGGGGCCTGCACCTCCACATCGTGCCCTGCAAGCACGTGCTCAGCTGGC
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CTGCTGCGGGCTGACTTCCCACTCTCTGTGGTCCAGCGAGAGCCGGCCAGGGACGCAGCCCCCAGCATCCCAGCCCCGGC
CGAGTGCCTGCCGGATGTGCAGGCCTGCACGGGCCCCACTTCCCCACACCTTGTCCTCTGGCACTACGACCCGCAGCGGG
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TGTGCCCGCGGCCCCGGCGACGCCTGCGTGCTGCCTGCCGTGCAGGGCCCCTGCCGGGGCTGGGAGCCGCGCTGGGCCTA
CAGCCCGCTGCTGCAGCAGTGCCATCCCTTCGTGTACGGTGGCTGCGAGGGCAACGGCAACAACTTCCACAGCCGCGAGA
GCTGCGAGGATGCCTGCCCCGTGCCGCGCACACCGCCCTGCCGCGCCTGCCGCCTCCGGAGCAAGCTGGCGCTGAGCCTG
TGCCGCAGCGACTTCGCCATCGTGGGGCGGCTCACGGAGGTGCTGGAGGAGCCCGAGGCCGCCGGCGGCATCGCCCGCGT
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GCAGGCCTGCGAGCTGCTCAACCGCTTCCAGGACTAGCCCCCGCAGGGGCCTGCGCCACCCCGTCCTGGTGAATAAACGC
ACTCCCTGTGCCTCAGAAAAAAAAAAAAAAAAAAAAA

EXHIBIT 2

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>zkun6
MPALRPLLPL
LLLLRLTSGAGLLPGLGSHPGVCPNQLSPNLWVDAQSTCERECSRDQDCAAAEKCCINVCGLHSCVAARF
PGSPAAPTTAASCEGFVCPQQGSDCDIWDGQPVCRCRDRCEKEPSFTCASDGLTYYNRCYMDAEACLRGL
HLHIVPCKHVLSWPPSSPGPPETTARPTPGAAPVPPALYSSPSPQAVQVGGTASLHCDVSGRPPPAVTWE
KQSHQRENLIMRPDQMYGNVVVTSIGQLVLYNARPEDAGLYTCTARNAAGLLRADFPLSVVQREPARDAA
PSIPAPAECLPDVQACTGPTSPHLVLWHYDPQRGGCMTFPARGCDGAARGFETYEACQQACARGPGDACV
LPAVQGPCRGWEPRWAYSPLLQQCHPFVYGGCEGNGNNFHSRESCEDACPVPRTPPCRACRLRSKLALSL
CRSDFAIVGRLTEVLEEPEAAGGIARVALEDVLKDDKMGLKFLGTKYLEVTLSGMDWACPCPNMTAGDGP
LVIMGEVRDGVAVLDAGSYVRAASEKRVKKILELLEKQACELLNRFQD

Multi-domain proteinase inhibitor zkun6

Darrell Conklin

Serine proteinase inhibitors regulate the proteolytic activity of target proteinases by occupying the active site and thereby preventing occupation by normal substrates. Although serine proteinase inhibitors fall into several unrelated structural classes, they all possess an exposed loop (variously termed an "inhibitor loop", a "reactive core", a "reactive site", a "binding loop") which is stabilized by intermolecular interactions between residues flanking the binding loop and the protein core (Bode and Huber, 1992). Interaction between inhibitor and enzyme produces a stable complex which disassociates very slowly, producing either virgin or a modified inhibitor which is cleaved at the scissile bond of the binding loop.

zkun6 encodes a multi-domain secreted protein of 548 amino acids.

Its domain structure can be described as follows. The domain boundaries should be considered approximate (e.g., +/- 5 residues).

domain	range	description
A	1-19	signal sequence
B	33-75	four-disulfide core proteinase inhibitor
C	93-157	follistatin-type proteinase inhibitor
D	203-286	I-set IG domain
E	299-351	kunitz proteinase inhibitor domain #1
F	359-409	kunitz proteinase inhibitor domain #2
G	412-548	frzb domain C-term domain

Domain A encodes a hydrophobic signal sequence which allows the zkun6 protein to be exported from the cell. Following this is a predominantly hydrophilic short linker domain which forms the N-terminus of the mature protein.

Domain B is predicted to fold into a "four-disulfide core" or Chelonianin type serine proteinase inhibitor domain. The Chelonianin family is characterized by a common structural motif which comprises two adjacent beta-hairpin motifs, each consisting of two antiparallel beta strands connected by a loop region. The secondary structure of this motif is depicted by beta-sheet topology K (Branden and Tooze, p. 28). The beta strands are linked by intra-chain hydrogen bonding and by a network of four disulfide bonds. These disulfide bonds stablize the structure of the proteinase inhibitor and render it less susceptible to degradation. This structural feature has caused the Chelonianin family to be referred to as the "four-disulfide core" family of proteinase inhibitors. This family includes human antileukoproteinase, human elafin, guinea pig caltrin-like protein, human kallman syndrome protein, sea turtle chelonianin, the mouse WDNM1 protein, and human epididymal secretory protein E4, and trout TOP-2, and C. Elegans C08G9. Several of these family members contain several copies of this structural motif. The four disulfide pairings in the B domain of zkun6 are: Cys33-Cys66, Cys49-Cys70, Cys53-Cys65, Cys49-Cys75.

Domain C is predicted to fold into a structure similar to that determined for the follistatin homology domain in SPARC (Swiss-Prot SPRC_HUMAN, PDB 1BMO, also known as BM-40 or osteonectin) (Hohenester

et al. 1997). This is a beta hairpin structure, followed by a small hydrophobic core of alpha/beta structure. Based on the disulfide bonding pattern in SPARC, we can infer the disulfide pairings in zkun6 as: Cys93-Cys105, Cys98-Cys114, Cys116-Cys146, Cys120-Cys139, and Cys128-Cys157.

The follistatin homology domain has substantial sequence similarity to the Kazal family (Bode and Huber 1992) of serine proteinase inhibitors. Based on analogy with the crystal structures for the proteinase inhibitors PEC-60 (PDB 1PCE), and ovomucoid (PDB 1OVO), the putative proteinase binding site in domain C of zkun6 comprises the residues Cys120 (P3), Glu121 (P2), Lys122 (P1), Glu123 (P1'), and Pro124 (P2'). The scissile bond of the binding loop will therefore reside between the P1 and P1' residues Lys122 and Glu123.

The D domain of zkun6 is predicted to fold into a structure similar to that determined for the telokin peptide (Swiss-Prot KMLS_HUMAN, PDB 1TLK). The telokin peptide falls into the class of immunoglobulins (Bork et al. 1994) which are all beta proteins folding into a beta-sandwich like structure. These have two beta sheets comprising 3+4 beta strands. Furthermore, the telokin peptide has been subclassified as an "I" set immunoglobulin domain. Other proteins with I set immunoglobulin domains include titin, vascular and neural cell adhesion molecules, and twitchin. In zkun6 there is a potential intra-domain D disulfide bond: Cys207-Cys263.

Domains E and F of zkun6 are predicted to fold into a Kunitz type serine proteinase inhibitor domain. The Kunitz domain is a folding domain of approximately 50-60 residues which forms a central anti-parallel beta sheet and a short C-terminal helix. The structure is stabilized by three disulfide bonds. Between the N-terminal region and the first beta strand resides the active inhibitory binding loop. This binding loop is disulfide bonded through the P2 Cys residue to the hairpin loop formed between the last two beta strands. In domain F, the protease binding loop (P3-P4') is expected to be comprised of the sequence 377-PCRGWE-372, with the P1 residue being Arg369, the P2 Cys residue at Cys378, and the P1' residue being Gly370.

Domain G has 27% identity to the C-terminal portion of a human frzb protein (Hu et al., 1998). The N-terminal or cysteine-rich portion of the frzb protein shows substantial similarity to the extracellular portion of the Drosophila "frizzled" protein, a potential seven transmembrane receptor. frzb is a secreted glycoprotein that modulates signaling activity of Wnt proteins. The N-terminal domain is sufficient for this biological activity (Lin et al., 1997), and the function of the C-terminal portion of frzb is unknown.

References

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